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USE OF AN ENTEROBACTERIUM OmpA PROTEIN ASSOCIATED WITH
THE ELAGIGILTV PEPTIDE, FOR TREATING MELANOMAS

The invention relates to the use of an enterobacterium,
5 in particular *Klebsiella pneumoniae*, OmpA membrane
protein, associated with an antigen or a hapten, for
preparing a pharmaceutical composition intended to
generate or increase a cytotoxic T response directed
10 against an infectious agent or a tumor cell. The
invention comprises the use of these compounds for
preventing and treating infection or cancer, in
particular cancers associated with a tumor antigen,
such as melanomas, and also for pharmaceutical
compositions comprising some of these compounds.

15 Vaccination is an effective means of preventing or
reducing viral or bacterial infections. The success of
vaccination campaigns in these domains has made it
possible to extend the vaccine concept, until now used
20 in the domain of infectology, to the domains of cancer
and of autoimmune diseases. Vaccinal antigens
administered alone to the host are often not
immunogenic enough to induce an immune response and
must, therefore, be associated with an adjuvant or
25 coupled to a carrier protein in order to induce (or
increase) the immunogenicity. Under these conditions,
only an immune response of the humoral type can be
induced. Now, in the context of antiviral therapy, the
generation of cytotoxic T lymphocytes (CTLs) capable of
30 recognizing and destroying the virus is of great
importance (Bachmann et al., 1994, Eur. J. Immunol.,
24, 2228-2236; Borrow P., 1997, J. Virol. Hepat., 4,
16-24), as attested by many studies showing, *in vivo*,
the protective role of responses directed against viral
35 epitopes (Arvin AM, 1992, J. Inf. Dis., 166, S 35-S41;
Koszinowski et al., 1987 Immunol. Lett., 16, 185-192).

The importance of CTL responses has also been greatly
documented in antitumor responses, in particular those

09/913107 "080901"

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corresponding to CTL epitopes and in the presence of such an adjuvant may lead to a state of specific tolerance, which may, in certain cases, produce the opposite effect to that desired, i.e. a decrease in the immune response (Toes et al., Proc. Nat. Acad. Sci. USA, 1996, 93, 7855-7860).

Thus, there exists, today, a great need for a compound which, when associated with a molecule, in particular an antigen or hapten, is capable of generating CTLs directed against said molecule. Such a compound could, in particular, be used for preparing a vaccinal composition intended to induce immune protection of the antiviral, antibacterial, antifungal, antiparasitic or antitumor CTL type.

Surprisingly, it has been demonstrated that an outer membrane protein of a gram-negative bacterium, in particular an enterobacterium OmpA protein such as the *Klebsiella pneumoniae* P40 protein (protein described in WO 95/27787 and WO 96/14415), has the property of eliciting a CTL response against a molecule which is covalently or noncovalently associated with it, preferably without having to add another adjuvant.

Thus, the present invention relates to the use of an enterobacterium OmpA protein, of a fragment thereof or of a nucleic acid sequence encoding said OmpA protein or a fragment thereof, for preparing a pharmaceutical composition intended to generate or increase a cytotoxic T response against an infectious agent or a tumor cell, *in vitro* or *in vivo*, preferably *in vivo*, and also for preparing a pharmaceutical composition intended to generate or increase said cytotoxic T response.

In the present invention, the term "protein" is intended to denote both peptides or polypeptides and

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the term "OmpA" (for "outer membrane protein") is intended to denote outer membrane proteins of the A type.

- 5 The expression "fragment of an OmpA protein" is intended to denote, in particular, any fragment of amino acid sequence included in the amino acid sequence of the OmpA protein which, when it is associated with an antigen or hapten specific for an infectious agent
10 or for a tumor cell, is capable of generating or increasing a cytotoxic T response directed against said infectious agent or said tumor cell, said fragment of the OmpA protein comprising at least 5 amino acids, preferably at least 10 amino acids or more preferably
15 at least 15 amino acids.

- The expression "antigen or hapten specific for an infectious agent or for a tumor cell" is intended to denote, in particular, any compound expressed by an
20 infectious agent, such as a virus, a bacterium, a yeast, a fungus or a parasite, or by a tumor cell, or a structural analog thereof, which, alone or in association with an adjuvant of immunity, is capable of inducing an immune response specific for said
25 infectious agent or for said tumor cell.

- In the present description, the expression "analog of an antigen or hapten" is intended to denote, in particular, a compound having structural similarity
30 with said antigen or hapten, capable of inducing an immunological response directed against said antigen or hapten in an organism immunized beforehand with said similar compound.

- 35 A subject of the invention is also the use as claimed in the invention, characterized in that said pharmaceutical composition also comprises, associated with said enterobacterium OmpA protein, an antigen or a

happen specific for said infectious agent or for said tumor cell.

5 Preferably, the invention comprises the use as claimed in the invention, characterized in that said infectious agent is a viral particle, a bacterium, a yeast, a fungus or a parasite.

10 In a particular embodiment, the invention comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium.

15 The methods for extracting bacterial membrane proteins are known to those skilled in the art and will not be developed in the present description. Mention may, for example, be made, but without being limited thereto, of the extraction method described by Haeuw J.H. et al. (Eur. J. Biochem, 255, 446-454, 1998).

20 In another preferred embodiment, the invention also comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained via the recombinant route.

25 30 The methods for preparing the recombinant proteins are, today, well known to those skilled in the art and will not be developed in the present description; reference may however be made to the method described in the examples. Among the cells which may be used for producing these recombinant proteins, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology

4:520-525), and also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4:558-563), and also insect cells in which methods may be used which implement, for example, baculoviruses (Luckow V.A., 1993, Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4:564-572).

Entirely preferably, the use as claimed in the invention is characterized in that the said enterobacterium is *Klebsiella pneumoniae*.

In particular, the invention relates to the use as claimed in the invention, characterized in that the amino acid sequence of said *Klebsiella pneumoniae* OmpA protein, or a fragment thereof, comprises:

- a) the amino acid sequence of sequence SEQ ID No. 2;
- b) the amino acid sequence of a sequence having at least 80%, preferably 90% and 95% homology, after optimal alignment, with the sequence SEQ ID No. 2; or
- c) the amino acid sequence of a fragment of at least 5 amino acids of a sequence as defined in a).

The expression "nucleic acid or amino acid sequence with at least 80% homology, after optimal alignment, with a given nucleic acid or amino acid sequence" is intended to denote a sequence which, after optimal alignment with said given sequence, comprises a percentage identity of at least 80% with said given sequence.

For the purposes of the present invention, the term "percentage identity" between two nucleic acid or amino

acid sequences is intended to denote the percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two nucleic acid or amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out by segment or by "window of comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, other than manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of the local homology algorithm of Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], by means of computer software which uses these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or with BLAST N or BLAST P comparison software).

The percentage identity between two nucleic acid or amino acid sequences is determined by comparing these two sequences which are optimally aligned by the window of comparison in which the region of the nucleic acid or amino acid sequence to be compared may comprise additions or deletions with respect to the reference sequence for optimal alignment between these two sequences. The percentage identity is calculated by determining the number of identical positions for which the nucleotide or the amino acid residue is identical between the two sequences, dividing this number of identical positions by the total number of positions in the window of comparison and multiplying the result

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Use may, for example, be made of the BLAST program "BLAST 2 sequences", which is available on the site <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>, the parameters used being those given by default (in particular for the "open gap penalty" parameter: 5, and the "extension gap penalty" parameter: 2; the matrix chosen being, for example, the "BLOSUM 62" matrix provided by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

The invention also comprises the use as claimed in the invention, characterized in that said antigen or hapten
25 is chosen from proteins, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing the CTL response against said infectious antigen or said tumor cell.

35 The invention also comprises the use as claimed in the invention, characterized in that said antigen or hapten is coupled by covalent attachment, in particular by chemical coupling, with said OmpA protein or a fragment thereof.

In a particular embodiment, the use as claimed in the invention is characterized in that one or more attachment elements is(are) introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate the chemical coupling; preferably, said attachment element introduced is an amino acid.

As claimed in the invention, it is possible to introduce one or more attachment elements, in particular amino acids, in order to facilitate the coupling reactions between the OmpA protein, or a fragment thereof, and said antigen or hapten. The covalent coupling between the OmpA protein, or a fragment thereof, and said antigen or hapten as claimed in the invention may be carried out at the N- or C-terminal end of the OmpA protein or a fragment thereof. The difunctional reagents which enable this coupling will be determined as a function of the end of the OmpA protein, or a fragment thereof, which is chosen for carrying out the coupling, and of the nature of said antigen or hapten to be coupled.

In another particular embodiment, the use as claimed in the invention is characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof, is produced by genetic recombination, when said antigen or hapten is peptide in nature.

The conjugates derived from coupling to said OmpA protein, or a fragment thereof, may be prepared by genetic recombination. The chimeric or hybrid protein (conjugate) may be produced using recombinant DNA techniques, by inserting or adding a sequence encoding said antigen or hapten which is peptide in nature into the DNA sequence encoding said OmpA protein or a fragment thereof.

The methods for synthesizing the hybrid molecules encompass the methods used in genetic engineering for constructing hybrid polynucleotides encoding desired polypeptide sequences. Advantageously, reference may, for example, be made to the technique for obtaining genes encoding fusion proteins, described by D.V. Goeddel (Gene expression technology, Methods in Enzymology, Vol. 185, 3-187, 1990).

- 10 In another aspect, the invention relates to the use as claimed in the invention, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding said hybrid protein, or comprises a vector containing a nucleic acid construct encoding said hybrid protein or a transformed host cell containing said nucleic acid construct, which is capable of expressing said hybrid protein.

- 20 The invention also comprises the use as claimed in the invention, for preparing a pharmaceutical composition intended to eliminate infectious agents or inhibit tumor growth.

- 25 Preferably, the use as claimed in the invention relates to the preparation of a pharmaceutical composition intended to prevent or treat infectious diseases or cancers, preferably cancers associated with a tumor antigen.

- 30 Among cancers in which the tumors express an associated tumor antigen, and which may be prevented or treated with the uses as claimed in the present invention, mention may be made, in particular, but without being limited thereto, of:

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- breast cancer, lung cancer, colon cancer and gastric carcinoma (Kawashima et al., 1999, Cancer Res. 59:431-5);

- mesothelioma, osteosarcoma, brain cancers (Xie et al., 1999, J. Natl. Cancer. Inst. 91:169-75);
- melanoma (Zheuten et al., 1998, Bratisl. Lek. Listy 99:426-34);
- 5 • cystic adinoma of the pancreas (Hammel et al., 1998, Eur. J. gastroenterol. Hepatol. 10:345-8);
- colorectal cancer (Ogura et al., 1998, Anticancer Res. 18:3669-75);
- renal cell carcinoma (Jantzer et al., 1998, Cancer
- 10 Res. 58:3078-86); and
- cancer of the ovary and of the cervix (Sonoda et al., 1996, Cancer. 77:1501-9).

15 A subject of the invention is in particular the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, for preparing a pharmaceutical immunization composition intended to prevent or treat an infectious disease, in particular of viral, bacterial, fungal or parasitic origin, or a

20 cancer, preferably associated with a tumor antigen, in particular melanomas.

The invention also comprises the use as claimed in the invention, characterized in that said pharmaceutical

25 composition is vehicled in a form which makes it possible to improve its stability and/or its immunogenicity, in particular in the form of a liposome.

30 Preferably, the invention comprises the use as claimed in the invention, characterized in that said vehicle is a viral vector containing a nucleic acid construct encoding said OmpA protein or a fragment thereof, said antigen or hapten, or said hybrid protein, or a

35 transformed host cell capable of expressing said OmpA protein or a fragment thereof, said antigen or hapten, or said hybrid protein.

The invention also comprises the use as claimed in the invention, characterized in that said nucleic acid construct, or the nucleic acid construct contained in said vector or said transformed host cell, comprises a
5 nucleic acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides, preferably 30 consecutive nucleotides, of the sequence SEQ ID No. 1, or a sequence having at least 80% homology, after optimal
10 alignment, with one of said sequences.

Preferably, a subject of the present invention is the use of an enterobacterium OmpA protein, or of a fragment thereof, associated with an antigen or a
15 hapten, for preparing a pharmaceutical composition intended to generate a cytotoxic T response directed against a tumor cell, as claimed in the present invention, characterized in that said antigen or hapten is the peptide of sequence SEQ ID No. 3: ELAGIGILTV and
20 in that the cytotoxic T response is directed against melanoma cells.

Also preferably, a subject of the present invention is the use of an enterobacterium OmpA protein, or a
25 fragment thereof, associated with the peptide of sequence ELAGIGILTV, as claimed in the present invention, for preparing a pharmaceutical composition intended to treat or prevent malignant melanomas.

30 In another aspect, a subject of the invention is a pharmaceutical composition as defined above, in particular:

- 35 - a pharmaceutical composition, characterized in that it comprises an enterobacterium OmpA protein, or a fragment thereof, associated, by mixing or by covalent coupling, with the peptide of sequence ELAGIGILTV; or
- a pharmaceutical composition, characterized in that it comprises a nucleic acid construct containing a

5 As defined above for the use as claimed in the invention, said pharmaceutical composition as claimed in the invention may, for example, comprise an enterobacterium OmpA protein, or a fragment thereof, coupled, by covalent attachment, to the peptide of
10 sequence ELAGIGILTV by chemical synthesis, using recombinant OmpA or OmpA obtained via an extraction method, or coupled by genetic recombination.

As also defined above for the use as claimed in the invention, said pharmaceutical composition as claimed in the invention may, for example, comprise a vector comprising a nucleic acid construct containing a nucleic acid encoding an enterobacterium OmpA protein, or a fragment thereof, and/or a nucleic acid encoding the peptide of sequence ELAGIGILTV, or alternatively a transformed cell capable of expressing an enterobacterium OmpA protein, or a fragment thereof, and/or the peptide of sequence ELAGIGILTV.

25 A subject of the invention is also a pharmaceutical composition, characterized in that it comprises the *Klebsiella pneumoniae* OmpA protein of sequence SEQ ID No. 2, a protein, the sequence of which has at least 80% homology, after optimal alignment, with the
30 sequence SEQ ID No. 2, or a fragment of at least 5 amino acids of said OmpA protein of sequence SEQ ID No. 2, associated, by mixing or by coupling, with the peptide of sequence ELAGIGILTV.

35 A subject of the present invention is also a pharmaceutical composition, characterized in that it comprises a nucleic acid construct containing a nucleic acid encoding the *Klebsiella pneumoniae* OmpA protein of sequence SEQ ID No. 2, a protein, the sequence of which

has at least 80% homology, after optimal alignment,
with the sequence SEQ ID No. 2, or a fragment of at
least 5 amino acids of said OmpA protein of sequence
SEQ ID No. 2, and a nucleic acid encoding the peptide
5 of sequence ELAGIGILTV.

According to the present invention, said compositions
will be vehicled in a form which makes it possible to
improve its stability and/or its immunogenicity, such
10 as in the form of a liposome, or of a viral vector or
of a transformed host cell capable of expressing said
OmpA protein, or a fragment thereof, and said peptide
of sequence ELAGIGILTV.

15 According to the present invention, said compositions
will preferably be contained in a pharmaceutically
acceptable medium.

For the purposes of the present invention, the
20 pharmaceutically acceptable medium is the medium in
which the compounds of the invention are administered,
preferably a medium which can be injected into humans.
It may consist of water, of an aqueous saline solution
or of an aqueous solution based on dextrose and/or on
25 glycerol.

According to the present invention, said compositions
may also contain a detergent.

30 The compositions as claimed in the invention may also
contain a detergent, and in particular any type of
pharmaceutically acceptable surfactant, such as for
example anionic, cationic, nonionic or amphoteric
surfactants. Use is preferably made of the detergents
35 Zwittergent 3-12 and octylglucopyranoside, and even
more preferably Zwittergent 3-14.

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Preferably, said pharmaceutical composition as claimed
5 in the invention contains no adjuvant of immunity,
besides the enterobacterium OmpA protein, or a fragment
thereof, or a nucleic acid encoding the enterobacterium
OmpA protein, or a fragment thereof, characteristic of
the pharmaceutical compositions of the invention.

The legends to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Figures 1A, 1B, 1C and 1D: Measurement of the anti-MELAN-A and anti-TRP-2 CTL activity of effector cells

20 After immunization with 50 μ g of hELA mixed with 3 μ g
of rP40 (figure 1A), 50 μ g of hELA mixed with 300 μ g of
rP40 (figure 1B), 50 μ g of hELA coupled to rP40
(figure 1C) or 50 μ g of the TRP-2 peptide mixed with
300 μ g of rP40 (figure 1D), the draining lymph node
25 cells are stimulated with EL-4 A2/Kb cells (figures 1A,
1B and 1C) or EL-4 cells (figure 1D) which had been
irradiated and prepulsed with 1 μ M of the relevant
peptide, before being evaluated for their capacity to
kill target cells which may (rectangle) or may not
30 (diamond) have been prepulsed with the relevant
peptide.

The X-axes of the points of figures 1A to 1D correspond to the ratio of the effector T cells (active lymphocytes) mixed together with the target cells (EL-4 A2/Kb or EL-4).

Figures 2A, 2B, 2C and 2D: Measurement of the anti-MELAN-A CTL activity of effector cells in the presence

of the rP40 protein compared to the CTL activity obtained with standard immunization protocol.

After immunization with hELA (50 µg) alone (ELA, figure 2A), hELA mixed with 300 µg of rP40 (ELA + P40, figure 2B), hELA coupled to 300 µg of rP40 (ELA/P40, figure 2C) or hELA mixed with 50 µg of P30 peptide adjuvanted with IFA (ELA + IFA + TT, figure 2D) (IFA for Incomplete Freund's Adjuvant and TT for Tetanus Toxoid), the draining lymph node cells are stimulated in vitro for two weeks with EL-4 A2/Kb cells which have been irradiated and prepulsed with 1 µM of the relevant peptide, before being evaluated for their capacity to kill EL-4 A2/Kb target cells which may (rectangle) or may not (diamond) have been prepulsed with the hELA peptide.

Example 1: Cloning of the gene encoding the *Klebsiella pneumoniae* P40 protein

The gene encoding the P40 protein was obtained by PCR amplification using the genomic DNA of *Klebsiella pneumoniae* IP 1145 (Nguyen et col., Gene, 1998). The gene fragment encoding this gene is inserted into various expression vectors under the control of various promoters, in particular that of the Trp operon. The nucleotide sequence and the peptide sequence of the P40 protein are represented by the sequences SEQ ID No. 1 and SEQ ID No. 2 hereinafter. An *E. coli* K12 producer strain was transformed with a pvaLP40 expression vector. The recombinant P40 protein (named rP40) is produced, in the form of inclusion bodies, with a considerable yield (> 10% in g of proteins/g of dry biomass).

This example is merely an illustration of the expression of the rP40 protein, this illustration possible being extended to other bacterial strains and to other expression vectors.

Example 2: Method for fermentation of rP40 fusion proteins

5 An Erlenmeyer flask containing 250 ml of TSB (Tryptic
Soy Broth, Difco) medium containing ampicilline
(100 µg/ml, Sigma) and tetracyclin (8 µg/ml, Sigma) is
inoculated with the transformed *E. coli* strain
described above. After overnight incubation at 37°C,
200 ml of this culture are used to seed 2 liters of
10 culture medium in a fermenter (Biolaaffite, France). In
a quite conventional way, the culture medium may be
composed of chemical agents supplemented with vitamins
and/or yeast extracts, which are known to promote high
density bacterial cell growth.

15 The parameters controlled during the fermentation are:
pH, stirring, temperature, level of oxygenation and
supply of combined sources (glycerol or glucose). In
general, the pH is regulated at 7.0 and the temperature
20 is fixed at 37°C. The growth is controlled by supplying
glycerol (87%) at a constant rate (12 ml/h) in order to
maintain the dissolved oxygen tension signal at 30%.
When the turbidity of the culture (measured at 580 nm)
reaches the value of 80 (after culturing for
25 approximately 24 hours), the protein production is
treated by adding indole acrylic acid (IAA) at the
final concentration of 25 mg/l. Approximately 4 hours
after induction, the cells are harvested by
centrifugation. The amount of wet biomass obtained is
30 approximately 200 g.

Example 3: Method for extracting and purifying the rP40 protein

Extracting the rP40

35 After centrifugation of the culture broth (4000 rpm,
10 min, 4°C), the cells are resuspended in a 25 mM
Tris-HCl buffer, pH 8.5. The insoluble components, or
inclusion bodies, are obtained after treatment with

lysozyme (0.5 g/liter, 1 hour at room temperature with gentle stirring). The inclusion body pellet obtained by centrifugation (15 min at 10,000 g at 4°C) is taken up in a 25 mM Tris-HCl buffer at pH 8.5 containing 5 mM
5 MgCl₂ and then centrifuged (15 min at 10,000 g).

The inclusion bodies are solubilized at 37°C for 2 hours in a 25 mM Tris-HCl buffer, pH 8.5, containing 7 M urea (denaturing agent) and 10 mM of dithiothreitol
10 (reduction of disulfide bridges). Centrifugation (15 min at 10,000 g) makes it possible to eliminate the insoluble particles.

This is then followed by resuspension in 13 volumes of
15 25 mM Tris-HCl buffer, pH 8.5, containing NaCl (8.76 g/l) and Zwittergent 3-14 (0.1%, w/v). The solution is left overnight at room temperature with gentle stirring in contact with the air (to promote renaturation of the protein by dilution and reoxidation
20 of the disulfide bridges).

Purifying the rP40 protein

- Anion exchange chromatography step

25 After a further centrifugation, the solution is dialyzed against a 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% Zwittergent 3-14 (100 volumes of buffer) overnight at 4°C.

30 The dialyzate is loaded on to a column containing a support of the strong anion exchanger type (Biorad Macro Prop High Q gel) equilibrated in the buffer described above, at a linear flow rate of 15 cm/h. The
35 proteins are detected at 280 nm. The rP40 protein is eluted, with a linear flow rate of 60 cm/h, for an NaCl concentration of 0.2 M in the 25 mM Tris-HCl buffer, pH 8.5: 0.1% Zwittergent 3-14.

- Cation exchange chromatography step

The fractions containing the rP40 protein are pooled and concentrated by ultrafiltration with the aid of an
5 Amicon cell system with stirring, used with a YM10-type Diaflo membrane (10 kDa cut-off threshold), for volumes of about 100 ml, or with the aid of a Millipore Minitan tangential flow filtration system, used with membrane plates having a 10 kDa cut-off threshold, for larger
10 volumes. The fraction thus concentrated is dialyzed overnight at 4°C against a 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14.

The dialyzate is loaded on to a column containing a
15 support of the strong cation exchanger type (Biorad Macro Prep High S gel) equilibrated in the 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14. The rP40 protein is eluted (rate 61 cm/h) for a 0.7 M NaCl concentration. The electrophoretic profiles
20 show about a 95% degree of purity. The condition of the protein is monitored by SDS-PAGE. The P40 protein, extracted from the *Klebsiella pneumoniae* membrane, has a characteristic electrophoretic (migration) behavior depending on whether it is in denatured or native form.
25 The native form (β -sheet structure) in fact has a lower molecular mass than the form which is denatured (α -helical structure) by the action of a denaturing agent, such as urea or guanidine hydrochloride, or by heating at 100°C in the presence of SDS. The rP40 protein is
30 not properly renatured at the end of renaturation, regardless of whether the latter is carried out in the presence or absence of 0.1% (w/v) Zwittergent 3-14. On the other hand, total renaturation is obtained after dialysis against a 25 mM Tris/HCl buffer, pH 8.5,
35 containing 0.1% (w/v) Zwittergent 3-14. However, it should be noted that this renaturation is only obtained when the dilution step and treatment at room temperature are, themselves, carried out in the

presence of Zwittergent 3-14 (negative results in the absence of detergent).

Example 4: Generation of CTLs

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The antitumor CTL responses directed against melanoma cells were defined for several antigens. These antigens are included in one of three categories:

- 10 a) rejection antigen specific for melanoma, such as those of the MAGE family (review by van der Bruggen et al., Science 254:1643);
- 15 b) antigens resulting from the mutation of normal proteins. This group includes MUM-1 (Coulie et al., Proc. Natl. Acad. Sci. USA 92:7976-7980 (1995)); CDK4 (Wolfel et al., Science 296:1281-1284 (1995)) and HLA-A2 (Brandel et al., J. Exp. Med. 183:2501-2508 (1996));
- 20 c) differentiation antigens expressed by melanomas and melanocytes. This group includes tyrosinase (Wolfel et al., Eur. J. Immunol. 4:759 (1994) and Brichard et al., Eur. J. Immunol. 26:224 (1996)); gp 100 (Kang et al., J. Immunol. 155:1343 (1995), Cox et al., Science 264:716 (1994), and Kawakami et al., J. Immunol. 155:3961 (1995)); gp75 (Wang et al., J. Exp. Med. 183:1131 (1996)), and Mart-1/MelanA (see US patent 5,620,886).
- 25

Of all these antigens, Mart-1/MelanA appears to be the best candidate for use in immunotherapy, this being for

30 several reasons. Firstly, this antigen was identified on the basis of the CTL response, in vivo, of the lymphocytes infiltrating the melanoma and not that, in vitro, of the peripheral blood cells, which would suggest greater relevance of this antigen in the

35 natural response, in vivo, against melanoma (Kawakami et al., J. Exp. Med. 180:347 (1994)). In addition, Mart-1/MelanA is expressed on all melanomas examined, which makes it a preferred target for intervention by immunotherapy. Finally, peptides derived from

Mart-1/MelanA are capable of inducing a specific CTL response in patients with melanoma expressing the HLA-A2 histocompatibility antigen (Rivoltini et al., J. Immunol. 154:2257 (1995); Valmori et al., J. Immunol. 160:1750 (1998)).

HLA-A2 is the most common allele expressed in Caucasians. The CTL epitopes of Mart-1/MelanA have been defined for this allele. The antigenic peptide recognized by the majority of human CTL lines comprises amino acids 27-35 AAGIGILTV (Kawakami et al., J. Exp. Med. 180:347 (1994)). In addition, studies on the affinity of binding with HLA-A*0201 and recognition by CTL clones have demonstrated that the optimum peptide for these two functions is the 26-35 decapeptide EAAGIGILTV (Romero et al., J. Immunol. 159:2366 (1997)). However, it appears that these peptides are weakly immunogenic in vitro (Valmori et al., J. Immunol. 160:1750 (1998)) and in vivo (Jaeger et al., Int. J. Cancer 66:162 (1996)).

When comparing the amino acid sequence of the T epitopes of Mart-1/MelanA with the peptide motifs of A*0201 (Rammensee et al., Immunogenetics 41:178 (1995)), it appears that the 26-35 and 27-35 peptides have nondominant anchoring residues at position 2 and therefore weakly bind the HLA-A*0201 molecule (Kawakami et al., J. Immunol. 154:3961 (1995)), which might explain their weak immunogenicity. The international patent application published under the number WO 98/58951 describes an analog to the 26-35 peptide, in which the alanine at position 2 has been replaced with a leucine (sequence which will be named ELA).

The hELA peptide, used in the experiments below, is the subject of patent application WO 98/58951 which is the property of the Institut Ludwig de Recherche sur le Cancer [Ludwig Cancer Research Institute]. hELA is an analog of the 26-35 decapeptide (EAAGIGILTV) of

Melan-A/MART-1, which is a protein expressed on melanocytes and melanomas. Although the 26-35 decapeptide of Melan-A/MART-1 is capable of binding to the HLA-A0201 molecule (Romero et al., 1997, J. Immunol. 159, 2366-2374), it is weakly immunogenic in vitro and in vivo (Valmori et al., 1998, J. Immunol. 160, 1750-1758). The hELA analog was generated by substituting the second amino acid of the 26-35 decapeptide of Melan-A/MART-1 (an alanine) with a leucine. The result of this substitution, which is based on analysis of the residues required for anchoring the peptides to the HLA-A0201 molecule, is more effective recognition by the CTLs of patients with melanoma and better immunogenicity in vitro (Valmori et al., 1998, J. Immunol. 160, 1750-1758). HLA-A*0201/Kb (A2/Kb) transgenic mice of the strain C57Bl/6 x BDA/2 (Vitiello et al., 1991, J. Exp. Med., 173, 1007-1015) were used in this study to test ELA. The class I MHC molecule expressed in these mice is a chimeric molecule made from the $\alpha 1$ and $\alpha 2$ domains of the human HLA-A0201 molecule (the most common allotype found) and from the $\alpha 3$ domain of the murine K^b molecule.

The TRP-2 peptide of sequence SEQ ID No. 4 is an octapeptide corresponding to amino acids 181-188 (VYDFFVWL) of tyrosinase-related protein 2 (TRP-2). TRP-2 is expressed in melanocytes and melanomas. It has been demonstrated that this antigen induces CTL responses which protect against melanoma in C57BL/6 (H-2K^b) mice (Bloom et al., 1997, J. Exp. Med. 185, 453-459).

A: Generation of anti-Melan-A and anti-TRP-2 CTLs after immunization with rP40 mixed with a peptide which is an analog to Melan-A or TRP-2

Experimental protocol

A2/Kb mice received, by subcutaneous injection at the base of the tail:

- 50 µg of ELA mixed with 3 or 300 µg of rP40;
- 50 µg of ELA covalently coupled to 300 µg of rP40.

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C57BL/6 mice received, by subcutaneous injection into the base of the tail:

- 50 µg of the TRP-2 peptide (181-188) mixed with 300 µg of rP40.

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Generation of cytotoxic effector cells

10 days after immunization, the mice are sacrificed and the lymphocytes from the draining lymph nodes are recovered in order to be stimulated, in vitro, with the relevant peptide.

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These lymphocytes (4 to 5×10^6) are cultured in a 24 well plate in DMEM plus 10 mM HEPES, 10% FCS and 50 µM β-2-mercaptoethanol, with 2 to 5×10^5 EL-4 A2/Kb cells or EL4 cells which have been irradiated (10 kRads) and prepulsed for 1 h at 37°C with 1 µM of the relevant peptide. After two weekly stimulations, the cells are assayed for their cytotoxic activity.

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Measurement of cytotoxic activity

The EL-4 A2/Kb cells or EL4 cells are incubated for 1 h with ^{51}Cr in the presence or absence of the relevant peptide, washed and then coincubated with the effector cells at various ratios, in a 96-well plate in a volume of 200 µl for 4 to 6 h at 37°C. The cells are then centrifuged and the ^{51}Cr release is measured in 100 µl of supernatant. The percentage of specific lysis is calculated as follows:

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$$\% \text{ specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100.$$

Results

TOP SECRET

As shown in figures 1A to 1D, the immunization of mice with an optimal dose of rP40 (300 µg) in a mixture with hELA (figure 1B) or TRP-2 (figure 1D) induces a strong specific CTL response. Such a response is also observed after immunization with rP40 coupled to hELA (figure 1C). On the other hand, the immunization with the peptide alone or rP40 alone (results not shown) or with the hELA peptide in a mixture with a suboptimal dose of rP40 (3 µg) does not induce any CTL activity (figure 1A). These results demonstrate that the rP40 molecule mixed with or coupled to immunogenic peptides makes it possible to induce a specific CTL response in vivo, this being without the addition of adjuvant.

B: Generation of anti-Melan-A CTLs after immunization with rP40 mixed with a peptide which is an analog to Melan-A, compared to a standard immunization protocol
Experimental protocol

A2/Kb mice received:

- 50 µl of IFA (incomplete Freud's adjuvant) by subcutaneous injection at the base of the tail, then, 3 weeks later, 50 µg of hELA in the presence of 50 µg of a helper-T p30 peptide derived from Tetanus Toxoid (TT) (Panina-Bordignon et al., Eur. J. Immunol., 1989, 19, 2237) adjuvanted with IFA. This protocol has been described for generating anti-peptide CTLs (Valmori et al., Eur. J. Immunol., 1994, 24, 1458) and is used as a positive control.
- 50 µg of hELA alone or 300 µg of rP40 mixed with or coupled to 50 µg of hELA.

Generation of cytotoxic effector cells

10 days after the final immunization, the mice are sacrificed and the lymphocytes from the draining lymph nodes are recovered in order to be stimulated, in vitro, with the relevant peptide.

These lymphocytes (4 to 5×10^6) are cultured in a 24-well plate in DMEM plus 10 mM HEPES, 10% FCS and 50 μ M β -2-mercaptoethanol, with 2 to 5×10^5 EL-4 A2/Kb cells (murine cells transfected with the HLA-A* 0201/Kb gene) which have been irradiated (10 kRads) and prepulsed for 1 h at 37°C with 1 μ M of the relevant peptide.

After one, two or three weekly stimulations, the cells are assayed for their cytotoxic activity.

The cytotoxic activity is measured according to the method described above.

Results

After immunization with nonadjuvanted rP40 coupled to hELA, an anti-hELA CTL activity comparable to that observed after immunization with hELA + P30/IFA is measured (cf. figures 2C and 2D). Similarly, the rP40 + hELA peptide mixture, itself also nonadjuvanted, generates CTLs in a way which is similar to that obtained with a conventional protocol for generating CTLs (cf. figures 2B and 2D).

No CTL activity was detected after immunization with the peptide alone (cf. figure 2A) or the rP40 protein alone (not shown), regardless of the day on which the effector cells were stimulated.

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